GROWTH INTERACTIONS OF ARTHROBACTER GLOBIFORMIS AND PSEUDOMONAS SP. IN RELATION TO THE RHIZOSPHERE EFFECT[1]

E. C. S. CHAN[2] AND H. KATZNELSON

Abstract

Studies on the influence of plant roots on the soil microflora have shown that the number of Gram-negative rods in the rhizosphere is relatively higher than the number of Gram-positive rods, coccoid rods, and sporeforming types. This ecological phenomenon was duplicated and studied in a model system using a *Pseudomonas* sp. as representative of the rhizosphere and *Arthrobacter globiformis* as representative of the soil flora. Growth of *A. globiformis* was strongly suppressed in the presence of the pseudomonad in root extracts of mature plants and in a medium containing casamino acids, yeast extract, glucose, and mineral salts (CAYG medium); suppression was less marked in soil extract. The pseudomonad was unaffected in the association. *A. globiformis* was inhibited in the first 48-hour incubation in CAYG medium by acid elaborated by the pseudomonad; the pH dropped to 5.3 in 16 hours but rose steadily to alkaline conditions after 48 hours, resulting in a delayed increase in the number of *A. globiformis* to approximately that of a pure culture, in 5 days. Under cultural conditions favoring pigment production by the pseudomonad, growth of *A. globiformis* was completely inhibited throughout this period. Another toxic principle was also produced by the pseudomonad. This substance was biologically active in mixed culture against the *Arthrobacter* but was present in low concentration.

Introduction

The ultimate problem in microbiology, according to Woods (9), is to explain in biochemical terms the life of microbes in the mixed association in which they exist in nature. Natural habitats are difficult to analyze because of the environmental variables and the heterogeneity of microbial species. Some simplification of experimental design is therefore necessary. One convenient and often valuable approach is that of a model system using mixtures of known cultures in the laboratory. Its advantage lies in making possible the analysis and synthesis of interactions under controlled conditions. In this sense, a microbial laboratory model is a useful tool in ecological studies to delineate the well-known phenomena of antibiosis, symbiosis, synergism, and commensalism.

The microbial flora of soils and rhizospheres has been extensively surveyed (1, 3, 5, 7, 8). These studies have shown that in the rhizosphere Gram-negative rods (represented by *Pseudomonas* species) are relatively more numerous than Gram-positive rods, coccoid rods, and sporeforming types (such as those belonging to the genera *Arthrobacter*, *Nocardia*, and *Bacillus*). In root-free soil, the latter groups predominate over the Gram-negative rods. The purpose of this investigation was to study the interaction of a representative rhizosphere organism and one from root-free soil in order to provide a rationale for the ecological observations noted.

[1]Manuscript received April 10, 1961.
Contribution No. 520, from the Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Canada.
[2]National Research Council Postdoctorate Fellow, in collaboration with the Canada Department of Agriculture, 1960–62.

Materials and Methods

Cultures

The organisms used were a *Pseudomonas* sp. isolated from the rhizosphere of wheat and *Arthrobacter globiformis* 425 (from the culture collection of the Microbiology Research Institute), a typical representative of the bacterial flora of the soil itself. Both organisms were able to develop together on prolonged incubation on crowded agar plates. The two species were easily differentiated by colonial characteristics (Fig. 1).

Culture Media

The media employed were root extracts, soil extract, and a complex medium prepared with dehydrated ingredients.

(*a*) Root extracts were prepared from mature soybean and barley plants. Roots were washed free of soil in running water and air-dried for about 12 hours. Eighteen grams of dried roots was soaked in 350 ml distilled water for $3\frac{1}{2}$ hours with occasional agitation, and the solution filtered through Whatman No. 12 paper. About 240 ml of extract (pH 6.4) was recovered. The extract was neutralized and then sterilized by autoclaving at 15 lb for 15 minutes.

(*b*) Soil extracts were prepared by adding 500 g of garden soil to 1 l. tap water and heating in an autoclave for 30–40 minutes at 15-lb pressure. The slurry was then filtered through Whatman No. 1 filter paper and the filtrate brought up to a final volume of 1 l. with tap water. The extract was autoclaved at 15 lb for 15 minutes (final pH, 6.2).

(*c*) The complex medium employed (CAYG) was of the following composition: casamino acids, 5 g; yeast extract (Nutritional Biochemicals Corp.), 5 g; glucose, 5 g; salt solution, 1 l., pH 6.8. The salt solution was made by adding to 900 ml of distilled water 10 ml of each of the stock salt solutions prepared by dissolving each of the following salts in 100 ml of distilled water: K_2HPO_4, 10 g; KNO_3, 5 g; $MgSO_4.7H_2O$, 2 g; $CaCl_2$, 1 g; NaCl, 1 g; $FeCl_3.6H_2O$, 0.1 g. The solution was adjusted to pH 7.0, heated to boiling, cooled, and filtered through Whatman No. 12 filter paper. The volume was adjusted to 1 l. for use in medium preparation.

For some experiments the medium was modified by reducing the glucose content to 0.1 and 0.01% (designated as $CAYG_{0.1}$ and $CAYG_{0.01}$, respectively).

Preparation of Inoculum

Inoculum was prepared by growing the organisms on slants of Difco Antibiotic Medium 2 (Bacto-Penassay Base Agar) for 24 hours at 25° C. Cells were transferred to fluid medium and the turbidity adjusted to a Klett reading of 51 using a red filter (No. 66). Of this suspension, 0.01 ml was used to inoculate 20 ml of medium.

Growth and pH Measurements

Growth of the pure and mixed cultures was followed by means of plate counts on Difco Antibiotic Medium 2. Pigments were not produced by the pseudomonad in this substrate. Serial dilutions were prepared by using Difco Antibiotic Medium 3 (Bacto-Penassay Broth) as the diluent since

PLATE I

FIG. 1. Colonial morphology of *Pseudomonas* sp. and *Arthrobacter globiformis*. The *Arthrobacter* colonies are smaller and can even develop on top of the *Pseudomonas* colonies on prolonged incubation.

preliminary experiments showed a rapid loss of viability of *A. globiformis* in distilled water. All pH values were determined with a Beckman pH meter. Plates and liquid cultures were incubated at $25 \pm 2°$ C; the latter were shaken at about 110 revolutions per minute on a rotary shaker.

Filtrate Preparation

Filtrates of the pseudomonad and of the mixed cultures were prepared by centrifugation of the cells at 12,100 *g* for 20 minutes in a Serval Superspeed centrifuge. Sterility was achieved by subsequent filtration through ultra-fine sintered-glass filters. Unless otherwise specified, all filtrates were adjusted to pH 7.2 before use.

Results

Model System with Root and Soil Extracts and Medium CAYG

When the pseudomonad and *A. globiformis* were grown together in mature soybean root extract, the latter organism was inhibited as shown in Fig. 2, whereas the former was unaffected. The same interaction pattern was also obtained with extracts of mature barley root. Pigment production by the pseudomonad did not occur in these extracts.

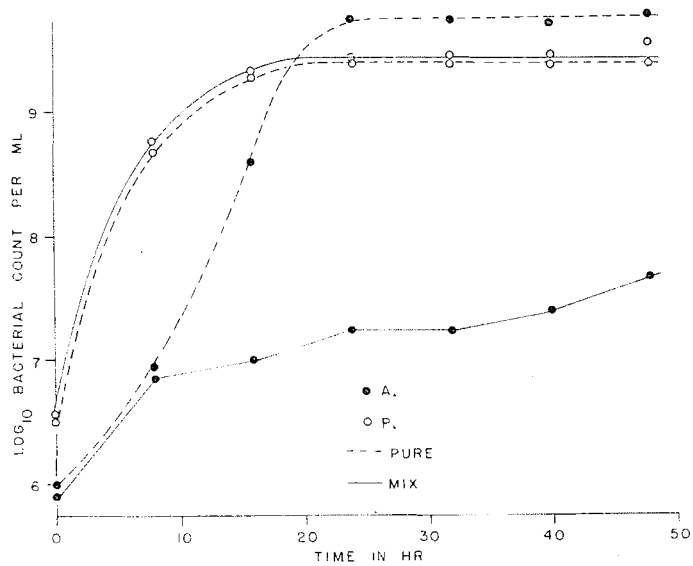

FIG. 2. Growth interaction of *Pseudomonas* sp. (P.) and *Arthrobacter globiformis* (A.) in mature soybean root extract.

Growth of a mixed culture of the two organisms in soil extract also proved unfavorable for *A. globiformis*; the pseudomonad was also non-pigmented in soil extract. The same general pattern of suppression of the *Arthrobacter* was observed as in Fig. 2 but this inhibition was very much less pronounced than in root extract. At the time of maximum inhibition (16 hours) the ratio of the number of pseudomonad to the number of *A. globiformis* in root extract of soybean was 213:1 whereas the ratio in soil extract was 4:1.

In CAYG medium, the *Arthrobacter* was also inhibited in mixed culture (Fig. 3) whereas the pseudomonad was unaffected and produced no pigment. The ratio of *Pseudomonas* to *Arthrobacter* was 9875:1. It may also be seen that the *Pseudomonas* has not or only a very short lag phase whereas the lag period of *A. globiformis* in pure culture is approximately 4 hours. In parallel studies it was found that the generation time of the pseudomonad in this medium is about 48 minutes while that for *A. globiformis* is about 84 minutes.

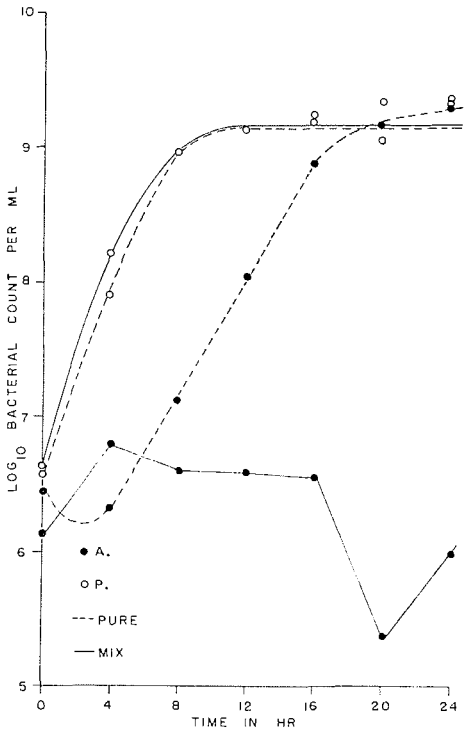

FIG. 3. Growth interaction of *Pseudomonas* sp. and *Arthrobacter globiformis* in a casamino acid – yeast extract – glucose – salts medium.

The effect of incubating a mixed culture in medium CAYG for an extended period is shown in Figs. 4 and 5. If Difco Bacto-yeast extract (Lot 437697) is used instead of N.B.C. yeast extract, the formation of a green fluorescent pigment by the *Pseudomonas* sp. is favored. (The routine use of N.B.C. yeast extract, Lot 7590, obviated this complication.) From Fig. 4 it can be seen that under these conditions growth of *A. globiformis* is suppressed throughout the 5 days of incubation, with no recovery. However, if pigment is not produced, counts of the *Arthrobacter* begin to increase on or about the second day so that its population finally approaches the level of the pure culture (Fig. 5).

The question remained concerning the factor(s) responsible for inhibition of *A. globiformis* in the absence of or before pigment formation. The possi-

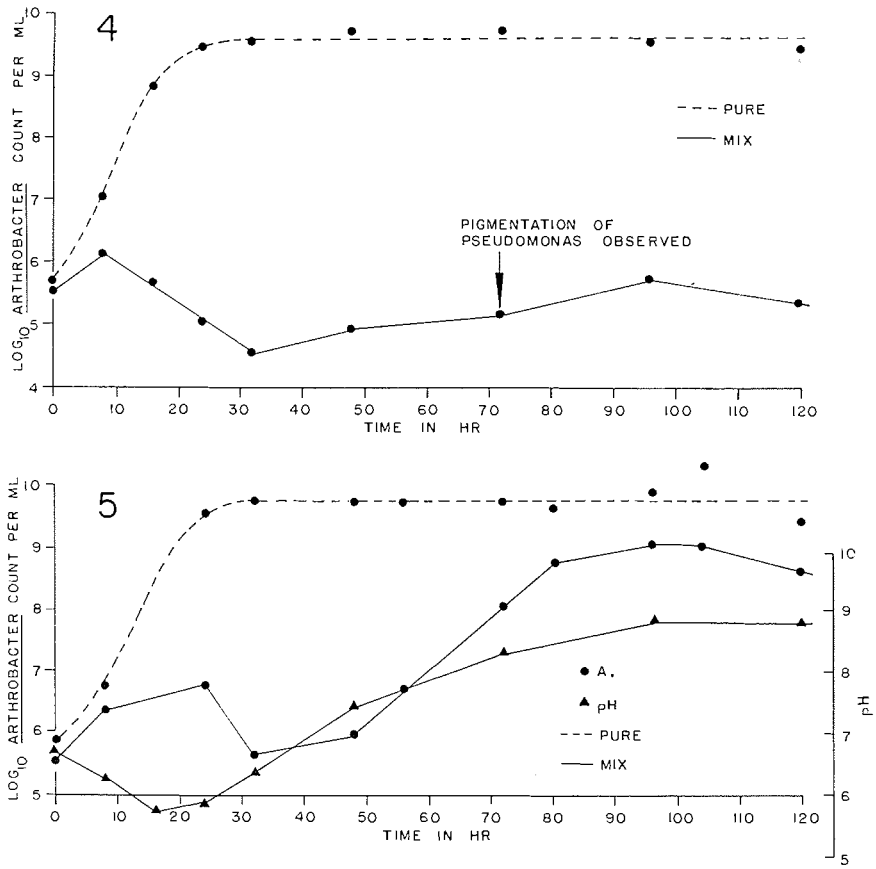

FIG. 4. Growth interaction of *Pseudomonas* sp. and *Arthrobacter globiformis* in medium CAYG in the presence of the green fluorescent pigment of the pseudomonad.

FIG. 5. Growth interaction of *Pseudomonas* sp. and *Arthrobacter globiformis* in medium CAYG in the absence of pseudomonad pigmentation.

bility existed that the production of acid by the pseudomonad in the presence of a carbohydrate might inhibit growth of the *Arthrobacter*. Therefore, pH determinations were made of a growing mixed culture at different times and the results obtained are also shown in Fig. 5. The growth curve of *A. globiformis* in mixed culture follows roughly the pH curve and suggests inhibition of *A. globiformis* by the pseudomonad as a result of acid production since a pure culture of the *Arthrobacter* becomes alkaline in this medium. Further experiments indicated that growth of *A. globiformis* is retarded when the initial pH is about 5.5; initial pH values from 6 to 9 do not affect the rate of growth. This is supporting evidence for the inhibition of this organism by acidity since the pH of the mixed culture may decrease to 5.3 in 16 hours. As final evidence of the pH effect, it was shown that the *Arthrobacter* is suppressed in a 16-hour unadjusted culture filtrate of the pseudomonad (pH 5.3). Raising the pH to 6.8 resulted in normal growth (Fig. 6).

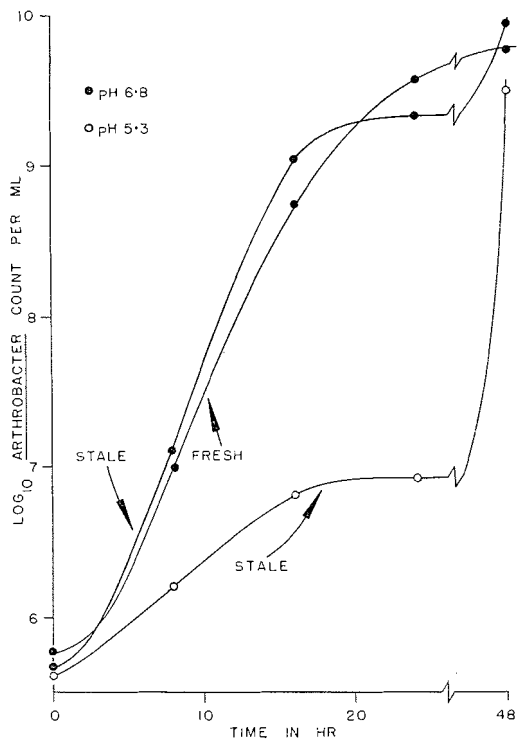

FIG. 6. Inhibition of *Arthrobacter globiformis* by the acid elaborated by *Pseudomonas* sp. *A. globiformis* was inoculated into sterile 16-hour pseudomonad filtrates with pH unadjusted, or adjusted to 6.8.

Additional Inhibitory Effects

In further experiments on the pH effect it was found that the addition of powdered $CaCO_3$ (0.1%) to a mixed culture raised the pH above 6.0, yet *A. globiformis* was still suppressed (Fig. 7). Furthermore, when the glucose in CAYG medium was reduced from 0.5% to 0.1 and 0.01%, the pH of a mixed culture did not drop below 6.5; nevertheless, as may be noted in Fig. 8, inhibition of *A. globiformis* was still marked. These results suggested the presence of other inhibitory factors in the system. Accordingly, growth of *A. globiformis* was followed in filtrates of the pure *Pseudomonas* culture and of mixed cultures using $CAYG_{0.01}$ broth. Inhibition occurred with 24-hour but not with 16-hour filtrates and indicated that the pseudomonad was again responsible for the effect. Table I shows the growth of *A. globiformis* in a 24-hour *Pseudomonas* culture filtrate of $CAYG_{0.01}$ broth. Dilution with distilled water resulted in increased growth of the organism, suggesting that a nutritional deficiency was not induced by the pseudomonad. This evidence further supports the hypothesis that an inhibitor is produced by the pseudomonad but only in low concentration at 24 hours, since slight dilution with fresh medium (or with water) overcomes the effect.

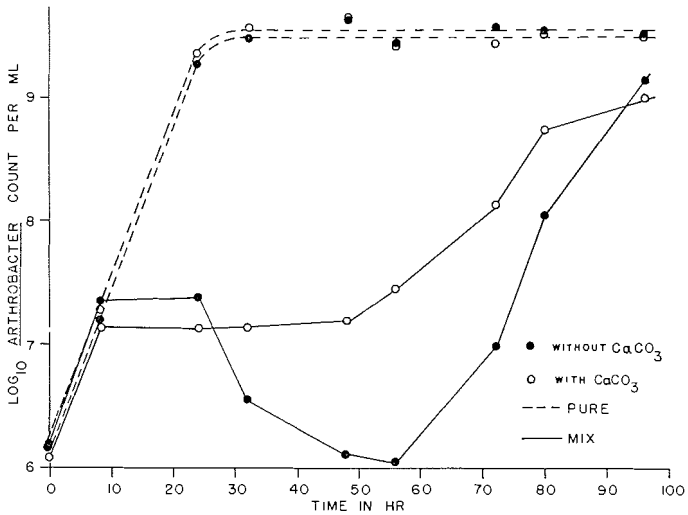

FIG. 7. Effect of calcium carbonate on the growth of *Arthrobacter globiformis* in mixed culture in medium CAYG.

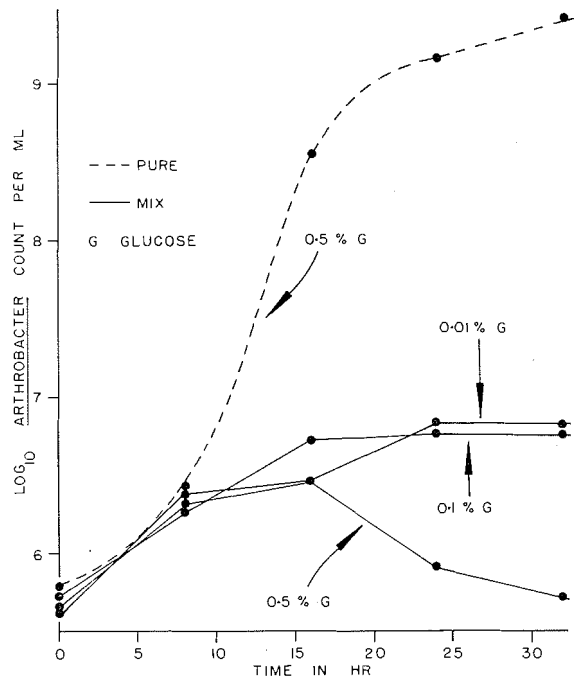

FIG. 8. Effect of reduced glucose in medium CAYG on the mixed culture growth of *Arthrobacter globiformis*.

TABLE I

Effect of dilution upon the growth of *Arthrobacter globiformis* in filtrate from a 24-hour culture of a *Pseudomonas* sp.

% Diluent	Diluent: Distilled water	Diluent: Fresh medium
0	37*	37
20	147	490
40	250	910
60	210	1740
80	440	1560
100	—	530

**A. globiformis* in millions per ml (after 24-hour incubation).

Discussion

The model system used in this investigation was designed to by-pass the difficulties inherent in any population analysis of a complex natural habitat. Although there are obvious dangers in such simplification, the results obtained with the mixed cultures reflect in some measure the microbiological events at the surface of a plant root, that is, the development of a bacterial flora in which Gram-negative rod-forming organisms predominate over Gram-positive rods, coccoid rods, and sporeforming types. In addition, the results provide a physiological basis for the observed shift in the microbial equilibrium of the soil as a result of penetration by a growing root. Population studies, as recorded in earlier publications (2, 4, 11), have all shown that the bacterial flora of the rhizosphere is more active metabolically and grows more rapidly than that of the root-free soil. The experiments reported herein not only yield further support for this but also provide additional possible reasons for the predominance of Gram-negative organisms as represented by *Pseudomonas* sp. in the root zone—acid production, pigment production, and liberation of inhibitory substances. It was significant that the inhibition of *Arthrobacter* in mixed culture in root extract was so much greater than in soil extract. The nutritional level of the latter in respect to amino nitrogen and sugars is very low and under such conditions the *Pseudomonas* does not develop normally, a situation which obtains in soil. The reverse effect must occur at the root surface or, as in the present experiments, in root extract, where sufficient nutrients are available to permit the *Pseudomonas* to grow rapidly.

The observation that only 24-hour and not 16-hour filtrates of pure *Pseudomonas* cultures (as well as mixed cultures) inhibited the *Arthrobacter* under conditions which precluded acid production is of interest in that it suggests the formation of still other inhibitory substances. Yet in mixed culture in the absence of acid production (Figs. 7 and 8) the *Arthrobacter* was inhibited as early as 8 hours after inoculation. A possible interpretation of this apparent discrepancy is that the inhibitory material was either of very low toxicity or was produced in such small amounts as not to be detectable in the culture filtrate before 24 hours. The early inhibition in mixed culture could be due to the intimate contact of the two organisms, which would permit one to affect

the other even though only minute amounts of toxic material were produced. Such a phenomenon may be effective also in natural habitats, such as soil, in which readily detectable amounts of antibiotic may not be formed (10). Antibiotic effects may nevertheless occur in various loci—in soil crumbs, on pieces of organic material—in which the intimate contact of microorganisms would render even extremely small amounts of inhibitory substances highly effective (6).

Acknowledgment

The senior author acknowledges the generous support of the National Research Council of Canada in a postdoctoral fellowship award. It is a pleasure also to acknowledge the cordiality shown him by the staff members of the Microbiology Research Institute, Canada Department of Agriculture, throughout this investigation in that laboratory.

References

1. CLARK, F. E. Notes on the types of bacteria associated with plant roots. Trans. Kansas Acad. Sci. **43**, 75–84 (1940).
2. KATZNELSON, H. and BOSE, B. Metabolic activity and phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere, and non-rhizosphere soil. Can. J. Microbiol. **5**, 79–85 (1959).
3. LOCHHEAD, A. G. Qualitative studies of soil microorganisms. III. Influence of plant growth on the character of the bacterial flora. Can. J. Research, C, **18**, 42–53 (1940).
4. ROUATT, J. W. and KATZNELSON, H. The comparative growth of bacterial isolates from rhizosphere and non-rhizosphere soils. Can. J. Microbiol. **3**, 271–275 (1957).
5. ROUATT, J. W. and KATZNELSON, H. A study of the bacteria on the root surface and in the rhizosphere soil of crop plants. J. Appl. Bacteriol. (1961, in press).
6. STEVENSON, I. L. Antibiotic activity of actinomycetes in soil as demonstrated by direct observation techniques. J. Gen. Microbiol. **15**, 372–380 (1956).
7. VAGNEROVA, K., MACURA, J., and CATSKA, V. Rhizosphere microflora of wheat. I. Composition and properties of bacterial flora during the first stages of wheat growth. Folia Microbiol. **5**, 298–310 (1960).
8. VOZNIAKOVSKAIA, I. M. and ZHIL'TSOVA, G. K. Species composition of some plants. Microbiology (U.S.S.R.), **27**, 599–605 (1957).
9. WOODS, D. D. The integration of research on the nutrition and metabolism of microorganisms. J. Gen. Microbiol. **9**, 151–173 (1953).
10. WRIGHT, J. M. The production of antibiotics in soil. 1. Production of gliotoxin by *Trichoderma viride*. Ann. Appl. Biol. **41**, 280–289 (1954).
11. ZAGALLO, A. C. and KATZNELSON, H. Metabolic activity of bacterial isolates from wheat rhizosphere and control soil. J. Bacteriol. **73**, 760–764 (1957).